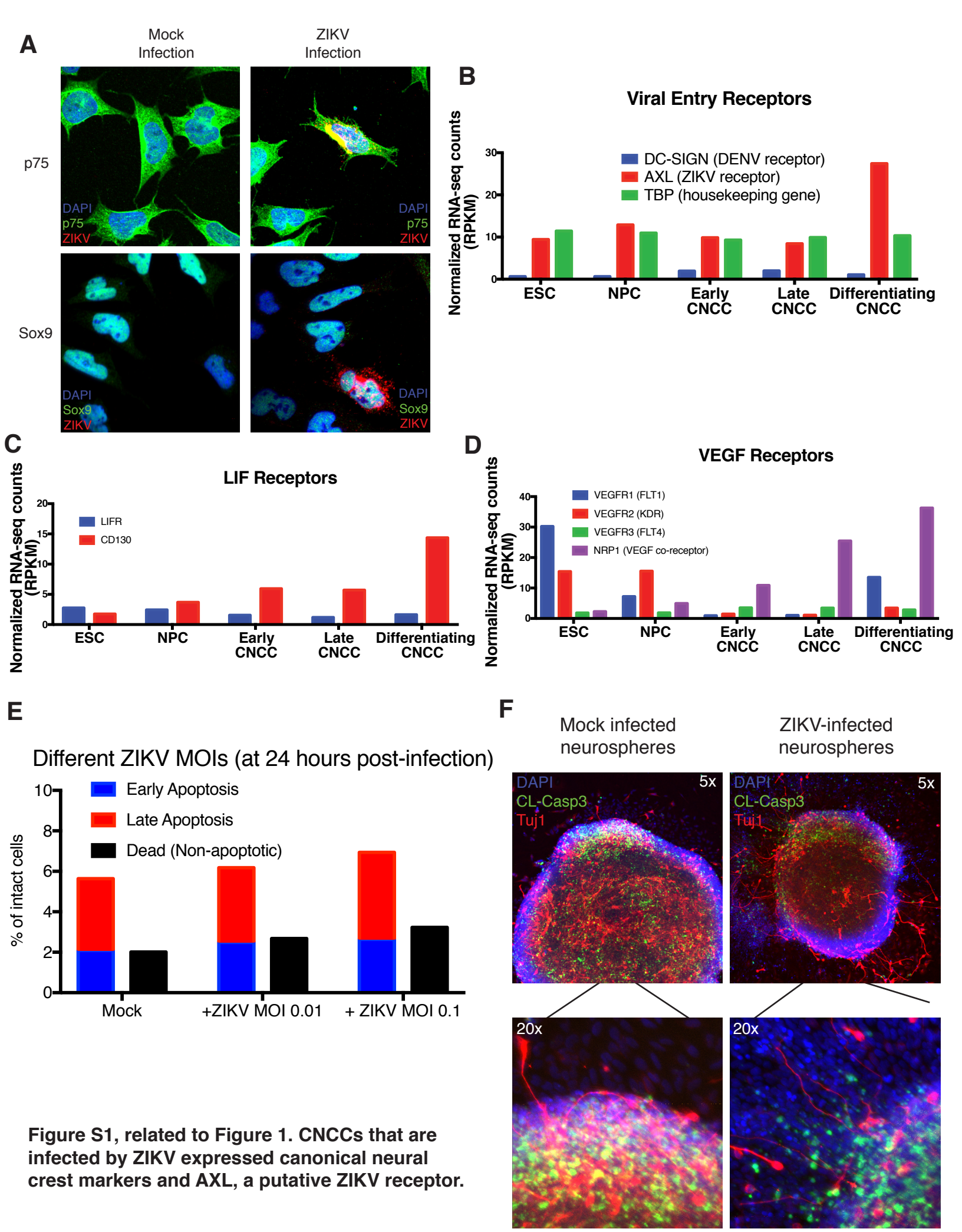

Figure S1, related to Figure 1. CNCCs that are infected by ZIKV expressed canonical neural crest markers and AXL, a putative ZIKV receptor.

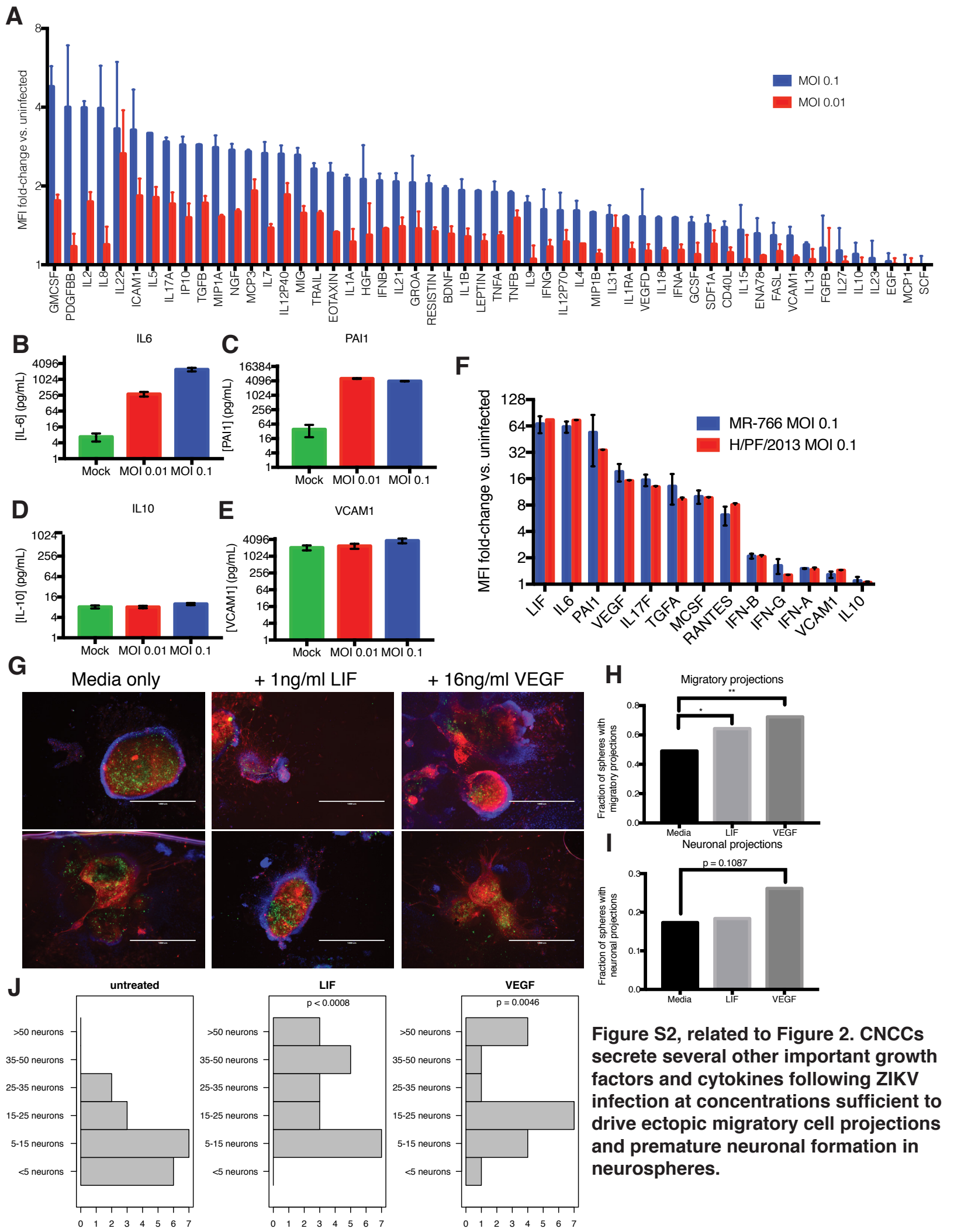

Figure S2, related to Figure 2. CNCCs secrete several other important growth factors and cytokines following ZIKV infection at concentrations sufficient to drive ectopic migratory cell projections and premature neuronal formation in neurospheres.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. CNCCs that are infected by ZIKV expressed canonical neural crest markers and AXL, a putative ZIKV receptor.
A. Confocal immunofluorescent images of (left) mock- and (right) ZIKV-infected CNCCs, stained for canonical neural crest lineage markers: (top) p75 and (bottom) Sox9 (green). Slides were also stained for flavivirus group antigen (red) and DAPI (blue).
B. mRNA expression data from published RNA-seq analysis for putative ZIKV and DENV viral entry receptors. (Rada-Iglesias et al., 2012)
C and D. mRNA expression data from published RNA-seq analysis for LIF (B) and VEGF (C) receptors. (Rada-Iglesias et al., 2012)
E. Quantifications of flow cytometry analysis of apoptotic death at 24 and 72 hours after infection. Cells were stained with Annexin V and propidium iodide to mark early apoptotic death (Annexin V+, PI-) to mark late apoptotic (Annexin V+/PI+) and non-apoptotic dead cells (Annexin V-/PI+).
F. Confocal immunofluorescent images of neurospheres 72 hours after mock or ZIKV infection. Neurospheres were stained for cleaved caspase-3 (green), Tuj1 (red), and DAPI (blue).

Figure S2, related to Figure 2. CNCCs secrete several other important growth factors and cytokines following ZIKV infection at concentrations sufficient to drive ectopic migratory cell projections and premature neuronal formation in neurospheres.
Cell culture supernatants were collected from CNCCs were infected with ZIKV, DENV, or mock infected for 24 hours. Secreted cytokine levels were measured by a 63-plex Luminex assay.
A. Cytokines and growth factors in CNCC culture supernatants following ZIKV (MR-766) infection. All analytes in the assay not displayed in Figure 2A are shown here. Data is represented as MFI fold-change compared to uninfected sample. Each bar represents mean MFI from two technical replicates from each of two independent biological experiments. Error bars represent S.D.
B-E. Concentrations of (B) IL-6, (C) PAI1, (D) IL-10, and (E) VCAM1 in culture supernatants after 24 hour ZIKV infection at MOI 0.1, 0.01 or mock infection. Values represent mean concentration as determined from standard curves for each analyte. Error bars represent S.E.M. of two independent biological experiments with two technical replicates each.
F. Cell culture supernatants were collected from CNCCs were infected with ZIKV (MR-766 or H/PF/2013) at MOI 0.1 or mock infected for 24 hours. Secreted cytokine levels were measured by a 63-plex Luminex assay. Shown here are analytes with >5-fold induction, IFNs, and VCAM1 and IL-10 for comparison. Data is represented as mean fluorescent intensity (MFI) fold-change compared to uninfected sample. Each bar represents mean MFI from two technical replicates from each of two independent biological experiments. Error bars represent S.D.
G. Additional neurosphere images from image set used for quantification in Figure 1F-G and Figure S2H-J. Seven days after sphere formation, exogenous LIF or VEGF was added to culture media, and spheres were observed for 3 days, followed by fixation and staining for DNA (DAPI, blue), apoptosis (cleaved caspase-3, green), and β-tubulin to mark neurons (Tuj1, red). Images are shown of neurospheres grown in culture media alone (left panels), with 1 ng/ml LIF (center panels), and with 16 ng/ml VEGF (right panels).
H and I. Images were scored by an objective observer for morphology. Data represents proportions of spheres showing (H) migratory cell projections, or (I) neuronal projections under normal media conditions and after the addition of LIF or VEGF.
J. Neurospheres were scored by an objective observer, and placed in bins based on the number of neuronal projections per neurosphere. LIF vs. media, $p < 8x10^{-4}$; VEGF vs. media, $p = 0.0046$, Wilcoxon signed-rank test.

SUPPLEMENTAL EXPERIMENTAL METHODS

Growth and titration of ZIKV stocks

Vero cells and ZIKV strain MR-766 (Uganda, 1947) were purchased from ATCC. DENV stocks were obtained as a gift from Dr. Eva Harris, and ZIKV H/PF/2013 stocks were obtained under MTA from the European Virus Archive, courtesy of Dr. Cécile Baronti. Vero cells were grown in DMEM (HyClone) supplemented with 10% fetal bovine serum and antibiotics (HyClone). ZIKV was inoculated into confluent Vero in in 75mm^2 flasks in 7ml of DMEM with 2% FBS for 1 hour, followed by addition of 8ml of DMEM with 10% FBS. Cells were observed to have cytopathic effects on day 4 following infection, and virus was subsequently harvested, spun at 1500 x *g* for 10 minutes to remove cell debris, and the clarified viral supernatant was aliquoted and frozen at -80°C.

Infectious viral particles were quantified using a focus forming assay (FFA). Briefly, 4 x 10^4 Vero cells were seeded in each well of a 96-well plate. Viral stocks were serially diluted in DMEM with 2% FBS, added to the Vero plates, and incubated for 2 hrs at 37°C. Following inoculation, 125μl of overlay [1% carboxymethylcellulose (Sigma), 1x MEM (Gibco), 20mM HEPES (Life Technologies) and 25mM sodium bicarbonate (Sigma)] was added to each well. Plates were incubated at 37°C for 48 hrs. Plates were then fixed with 1% paraformaldehyde (Electron Microscopy Sciences) for 1 hr, and washed 3x with PBS. Cells were permeabilized in saponin buffer solution [PBS with 0.1% saponin (Sigma) and 0.1% BSA] for 5 minutes, followed by staining at room temperature with anti-flavivirus group antigen antibody (Clone 4G2, Millipore) at 500ng/ml in saponin buffer for 2 hrs. Plates were 3x washed with 0.05% Tween (Sigma) in PBS, followed by staining at room temperature with anti-mouse-HRP in saponin buffer solution for 2 hrs. Plates were washed again 3x, and 50μl TrueBlue substrate (KPL) was added to each well. Plates were rocked until visible plaques developed, then washed 4x with deionized water. Plaques were counted, and viral stock concentrations calculated based on dilution series.

Tissue culture of hESCs, neurospheres and CNCCs

Pluripotent lines were differentiated into CNCC as previously described (Rada-Iglesias et al., 2011). Briefly, hESCs/iPSCs were incubated with 2mg/ml collagenase. Once detached, clusters of 100-200 cells were plated in CNCC differentiation medium: 1:1 Neurobasal medium/D-MEM F-12 medium (Invitrogen), 0.5× B-27 supplement with Vitamin A (50× stock, Invitrogen), 0.5× N-2 supplement (100× stock, Invitrogen), 20 ng/ml bFGF (Peprotech), 20 ng/ml EGF (Sigma-Aldrich), 5 μg/ml bovine insulin (Sigma-Aldrich) and 1× Glutamax-I supplement (100× stock, Invitrogen). Medium was changed every other day. After seven days of differentiation, neuroepithelial spheres attached to the dish and gave rise to migratory CNCC. Three-four days after the appearance of the first CNCC, cells were dissociated with accutase until single cells and passaged onto fibronectin-coated plates. CNCCs were then transitioned to CNCC early maintenance media: 1:1 Neurobasal medium/D-MEM F-12 medium (Invitrogen), 0.5× B-27 supplement with Vitamin A (50× stock, Invitrogen), 0.5× N-2 supplement (100× stock, Invitrogen), 20 ng/ml bFGF (Peprotech), 20 ng/ml EGF (Sigma-Aldrich), 1 mg/ml bovine serum albumin, serum replacement grade (Gemini Bio-Products # 700-104P) and 1× Glutamax-I supplement (100× stock, Invitrogen). CNCCs were passaged onto fibronectin-coated plates 1:3 every three days, and after 2-3 passages, transitioned to CNCC long term maintenance media, which is composed of CNCC early maintenance media plus 3uM ChIRON 99021 (Selleck, CHIR-99021) and 50pg/ml BMP2 (Peprotech). Cells were maintained on fibronectin with passaging every ~3 days, and collected at passage 3-6 for all downstream assays.

ZIKV infections and flow cytometry

hESC monolayers, CNCC monolayers or neurospheres in suspension were washed once in PBS, then ZIKV was added at matched MOI in a total volume of 1ml. Samples were incubated at 37°C for 1 hr, with manual plate swirling every 10 min. Inoculate was then aspirated and replaced with 2ml normal culture media, and cells were incubated for 23 hrs.

CNCC and hESC monolayers were washed twice with PBS. Neurospheres were allowed to settle by gravity, culture media was aspirated, and neurospheres were resuspended in 1ml of PBS, followed by a repeated wash. Following washing, 1mL of Accutase (Life Technologies) was added to each neurosphere pellet or monolayer well, and incubated for 3-5 minutes, until detachment to single cells was observed. Samples were then diluted in 9ml of FACS staining buffer (PBS with 2% FBS) and centrifuged at 1200rpm for 10 minutes. Pellets were resuspended and washed again in 10mL FACS staining buffer. Samples were then fixed for 10 minutes in FacsLyse (BD, 10x concentrate diluted 1:10 in water) and permeabilized for 10 minutes in Perm Buffer II (BD, 10x concentrate diluted 1:10 in water). Samples were washed twice in FACS staining buffer, then were stained with 1:1000 anti-flavivirus group antigen antibody (Clone 4G2, Millipore) directly conjugated to AlexaFluor 488 (Abcam). Cells were measured on a MACSQuant flow cytometer (Miltenyi) and analyzed in FlowJo v10.1 (Treestar). Statistics were calculated using Prism 7 (Graphpad Sotware).

Transwell co-incubation assay

CNCCs were seeded on a 0.4μm transwell membrane (Corning), and infected with ZIKV (or mock infected) for 1 hour in PBS. Transwell were then washed with PBS, following by replacement with 0.5ml normal CNCC media (described above) and, after 24 hours, were transferred into wells containing neurospheres in 1.5ml media. The plate was co-incubated at 37°C for 72 hours, followed by fixation and immunostaining for confocal microscopy. Cleaved caspase-3 antibody (clone #9661, Cell Signaling Technology) was used at a 1:1000 dilution with staining for 12-16 hours; Tubb3 (clone Tuj1, Biolegend #801201) was used at a 1:500 dilution with staining for 12-16 hours; ZIKV/Pan-flavivirus antibody (clone D1-4G2-4-15, Millipore) was used at a 1:500 dilution with staining for 1-2 hours. Appropriate Alexa 488, or Alexa 647, labeled secondary antibodies and/or DAPI counterstaining was used for visualization on a confocal microscope (Leica TSC SP2) at 5X and 20X magnifications. The CNCCs were separately analyzed by flow cytometry using the anti-flavivirus group antigen antibody to confirm positive infection. Images were scored by a trained, blinded observer for morphology. Specifically, images were scored for having neuronal projections, migratory cell projections, or no projections.

62-plex Human Cytokine Immunoassay

This assay was performed at the Human Immune Monitoring Center at Stanford University with human cytokine/chemokine 62-plex (eBiosciences/Affymetrix). Briefly, beads were added to a 96 well plate and washed in a Biotek ELx405 washer. Samples (25μl of conditioned media from 2ml total culture supernatant, harvested 24 hours after ZIKV, DENV, or mock infection) were added to the plate containing the mixed antibody-linked beads and incubated at room temperature for 1 hour followed by overnight incubation at 4°C with shaking. Cold and room temperature incubation steps were performed on an orbital shaker at 500-600 rpm. Following the overnight incubation, plates were washed in a Biotek ELx405 washer and then biotinylated detection antibody added for 60 minutes at room temperature with shaking. The plate was washed as above and streptavidin-PE was added. After incubation for 30 minutes at room temperature, wash was performed as above and reading buffer was added to the wells. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a

lower bound of 50 beads per sample per cytokine. Custom assay Control beads by Radix Biosolutions were added to all wells.

Immunofluorescent staining and image analysis
Cells were fixed in fresh 4% paraformaldehyde. For staining with antibodies recognizing intracellular epitopes, phosphate buffered saline with 0.5 mg/ml BSA and 0.1% Triton X-100 was used for blocking and permeabilization. Cleaved caspase-3 antibody (clone #9661, Cell Signaling Technology) was used at a 1:1000 dilution with staining for 12-16 hours; Tubb3 (clone Tuj1, Biolegend #801201) was used at a 1:500 dilution with staining for 12-16 hours; ZIKV/Pan-flavivirus antibody (clone D1-4G2-4-15, Millipore) was used at a 1:500 dilution with staining for 1-2 hours. Appropriate Alexa 488, or Alexa 647, labeled secondary antibodies and/or DAPI counterstaining was used for visualization on a confocal microscope (Leica TSC SP2) at 5X and 20X magnifications. Images for blinded morphological analysis were taken on an epifluorescence microscrope (EVOS, AMG).

Image analysis of neurosphere morphology
Z-stacks were taken on the Leica TSC SP2 confocal microscope at 10μm intervals. Image analysis was done in ImageJ, where a composite of the DAPI, Tuj1, and cleaved-caspase3 channels was made and the maximum projection of the stacks was taken for both 5X and 20X magnifications. 155 images taken on an epifluorescence microscope with only the Tuj1 channel were randomly assigned numbers and sent to a trained, blinded analyst. Each sphere was determined to have (a) no projections, (b) migratory cells, (c) neuronal projections or both (b) migratory cells and (c) neuronal projections. For the images with neuronal projections, the approximate number of neuronal projections were also quantified as <5 neurons, 5-15 neurons, 15-25 neurons, 25-35 neurons, 35-50 neurons, or >50 neurons. Plots were made and statistics were calculated using Prism 7 (Graphpad Sotware).

Image analysis of cleaved caspase-3 activity
For quantification of caspase signal, Z-stacks were taken on a Leica TSC SP2 confocal microscope at 10μm intervals. The 3 sections with the greatest antibody penetrance were utilized. In ImageJ, for both CL-caspase3 and Tuj1 staining, binary masks using OTSU were made for each section. The areas of these binary masks were then quantified in ImageJ. A ratio of the caspase signal to the Tuj1 signal was taken to further control for antibody penetrance. The average of these ratios was plotted and statistics were calculated using Prism 7 (Graphpad Software).

SUPPLEMENTAL REFERENCES

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